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In vitro squelching of activated transcription by serum response factor: evidence for a common coactivator used by multiple transcriptional activators

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ABSTRACT

Low amounts of serum response factor (SRF) activate transcription *in vitro* from a fos promoter construct containing an SRF binding site. Using this human HeLa cell-derived *in vitro* transcription system, we have found that high amounts of SRF inhibited, or 'squelched', transcription from this construct. Transcription from several other promoters activated by different gene-specific factors, including CREB and the acidic activator VP16, was also inhibited by high amounts of SRF. Basal transcription, from TATA-only promoters, however, was not inhibited. These results suggest that SRF binds to a common factor(s) (termed coactivator) required for activated transcription by a diverse group of transcriptional activators. Inhibition of transcription by SRF could be blocked by a double stranded oligonucleotide containing an SRF binding site. Mutations in SRF which abolished its DNA binding activity also reduced its ability to inhibit transcription. In addition, a C-terminal truncation of SRF which reduced its ability to activate transcription also reduced SRF's ability to inhibit transcription. These results suggest that activation and inhibition of transcription may be mediated by SRF binding to the same factor and that SRF can only bind to this factor when SRF is bound to plasmid DNA.

INTRODUCTION

Considerable progress has been made in identifying transcription factors which bind to promoters and enhancers and thereby activate transcription from specific genes (reviewed in ref. 1). The mechanism by which these gene-specific transcription factors activate the general transcription machinery is unclear. The general transcription factors consist of RNA polymerase II, TFIIA, -B, -D, -E, -F and -G (reviewed in ref. 2; 3) and are all required for 'basal' transcription from TATA-only promoters. The basal transcription level can be increased to an 'activated' level by gene-specific factors when the promoter contains their specific binding sites. These gene-specific factors fall into specific classes based on the mapping of their transcriptional activation domains. These include acidic, glutamine-rich, and proline-rich activation domains although the activation domains of many transcription factors have yet to be defined (reviewed in ref. 1).

It is possible that the gene-specific factors interact directly with one of the general transcription factors such that transcriptional initiation is increased. This is supported by the observation that TFIID, the TATA binding factor, and TFIIB can be retained on an affinity column containing the herpes viral transcriptional activator VP16 (4, 5). VP16 is one of the most potent transcriptional activators and contains an acidic activation domain (6, 7).

Alternatively, there may be an additional factor(s) which is required for the effect of the gene-specific transcription factors. Such a factor, termed a coactivator, adaptor or mediator, would be required for activated but not basal transcription (8–10). This is supported by several lines of evidence. First, this 'coactivator' can be titered out, or 'squelched', *in vivo* by expression of high amounts of a gene-specific transcription factor. These specific transcription factors inhibited expression from genes lacking their binding site (7,11,12). Only certain genes were inhibited in this fashion suggesting that there may be multiple targets for gene-specific activators (13,14) and that different classes of transcriptional activators may function through distinct coactivators. Second, similar experiments were performed *in vitro* where high levels of GAL4-VP16 inhibited transcription from a promoter containing a GAL4 binding site as well as from a heterologous promoter (9, 10). In the presence of an oligonucleotide containing a GAL4 binding site, GAL4-VP16 inhibited activated but not basal transcription (9) demonstrating that under these conditions a general factor was not being titered out. In addition, a partially purified yeast fraction, presumably containing the coactivator, was able to relieve the inhibition by GAL4-VP16, while RNA polymerase II and the other general transcription factors had no effect (10). Recently, a coactivator has been further purified from yeast extracts and found to be required for activation of transcription by GAL4-VP16 and the yeast activator GCN4 (15).

The third line of evidence for a coactivator comes from the cloning of TFIID from yeast, drosophila and humans (16–23). Recombinant TFIID could substitute for TFIID purified from HeLa cells to give basal transcription. This level of transcription, however, could not be increased further by gene-specific transcriptional activators such as SP1 and USF (8, 21). This has been interpreted as being due to the lack of a coactivator from the cloned TFIID preparation while the coactivator is normally present in TFIID preparations from HeLa cells (8). Recently,

distinct coactivators have been identified that separate from TFIID upon purification but these factors did not completely restore function when combined with recombinant TFIID (24,25).

Serum response factor (SRF) is a nuclear factor which binds with high affinity to the serum response element (SRE)(26–28). This element is the key sequence for the rapid transcriptional activation of the c-fos promoter in cells treated with serum and growth factors (29 and reviewed in ref. 30). SRF has been purified and cloned (31–34). It is a 508 amino acid protein which binds DNA as a dimer. The DNA binding and dimerization domains have been localized to the center of the protein but have no clear similarity to motifs known in other DNA binding proteins (34). SRF does, however, represent a class of DNA binding proteins as a number of yeast and plant genes (MCM1, ARG80, agamous, deficiens) have been cloned that have homology to SRF's DNA binding domain (34–36). SRF also does not contain a region with homology to known transcriptional activation domains. SRF does, however, activate transcription *in vitro* from SRE containing templates suggesting that it is a positively acting transcription factor (34,37).

We have previously found that activation of transcription by SRF may be due to its effects on TFIID function. SRF could only activate transcription when bound to DNA before or in the presence of TFIID. If TFIID was bound to DNA first, subsequently added SRF could no longer activate transcription efficiently (38). These results suggested that SRF causes TFIID to adopt an active conformation and that when TFIID binds first it adopts an inactive conformation that can no longer be affected by SRF. This effect may not be direct because, as with SP1 and USF, SRF was not able to activate transcription *in vitro* when bacterially made TFIID was used instead of native TFIID (38). Thus, SRF may interact with a coactivator to mediate its effect on TFIID. We were also interested in determining whether SRF uses the same or distinct coactivator(s) as other gene-specific factors. To develop an assay for such a coactivator, we have used high amounts of SRF to titrate out a coactivator(s) from the transcription reaction and have found that activated but not basal transcription was inhibited. Previous analyses of inhibition of transcription *in vitro* have used yeast derived systems (9,10) while we have used human HeLa cell-derived transcription factors with significantly different results. We describe here the conditions and characteristics of this inhibition by high levels of SRF.

METHODS

Plasmids

The fosCAT plasmids, pFC53, pFC53X, pFC72 and the internal control plasmid pML(s) were as previously described (37,39). pFC53G contains one GAL4 binding site cloned at −53 in pFC53. It was constructed by cloning the oligonucleotide TCGAGCGGAGGACTGTCCTCCGC into the XhoI site of pFC53. pMC50 was derived from pML(s) and pFC53 by digesting pML(s) with PvuII, adding an XbaI linker and digesting with SmaI and XbaI. This gave a fragment containing −50 to +30 of the adenovirus major late promoter. This was cloned in place of the fos promoter into pFC53 by digesting pFC53 with BglII, blunting this end with Klenow fragment of DNA polymerase I, digesting with XbaI, and isolating the large fragment produced.

SRF bacterial expression vectors were as described (40). BSRF-In171 was generated from pARSRF-Nde (w.t. SRF) by inserting a 12-mer XhoI linker (New Engand Biolabs) at the StuI site of SRF. BSRF-In206 was generated similarly into the BclI site of SRF, except that the BclI site was first blunted with Klenow fragment of DNA polymerase I and an 8-mer XhoI linker was inserted. BSRFΔ412-508 was generated from pSRF-Nde by deletion of an SphI to BamHI fragment and insertion of an XbaI termination linker (New Engand Biolabs). BSRFΔ1-114 was generated by digesting pARSRF-Nde with ApaI, blunting the ends with T4 DNA polymerase, ligating on BamHI 10-mer linkers, digesting with BamHI and gel purifying a 1.2 kb band. This band was cloned into pAR3040 at the BamHI site. This resulted in expression of 11 amino acids of the T7 phage gene 10 protein fused to amino acids 115 to 508 of SRF.

Mutant SRFpm143-6 was made by site directed mutagenesis of pARSRF-Nde as described (41) using the oligonucleotide CCCGGGGCCTCGTGGCCGGCAAGATGGAGTTC where the underlined nucleotides are changes from wt SRF. This mutation results in the change of amino acids 143, 145, and 146 from arginine, lysine, and isoleucine to leucine, alanine, and glycine, respectively.

The oligonucleotides used, XGL and XGLM, were as described (40).

Purification of SRF

SRF was isolated from bacteria containing the SRF expression vector pARSRF as previously described (40). SRF was purified by electroelution from SDS-polyacrylamide gels as described (40) except that after electroelution, SRF was precipitated with four volumes of cold acetone for 30 minutes in a dry ice/ethanol bath and pelleted by centrifugation at 10,000g for 20 minutes. The pellet was washed with 80% acetone to remove residual SDS. The pellet was then lyophilized, denatured in guanidine and renatured as described (40). Control protein was purified from E. coli containing pAR3040, the parental vector plasmid of pARSRF, by cutting a gel slice from the same region as SRF normally migrates, and purifying the protein as described above. The amounts of purified w.t. and mutant SRFs were quantitated by electrophoresis on SDS-polyacrylamide gels, coomasie blue staining, and comparison to standards of bovine serum albumin. We were typically able to prepare SRF at a concentration of 50 to 100 μg/ml (0.8 to 1.6 μM). SRF was insoluble at higher concentrations. Where possible, mutants were compared to wild type protein in immunoblots using SRF specific sera (41).

GAL4-VP16 was was purified from bacteria as described (42) and was a kind gift from Jerry Workman.

Fractionation of the general transcription factors

RNA polymerase II, TFIIA, -B, -D, -E, and -F were partially purified from HeLa cell nuclear extracts into three fractions as previously described (40). The nuclear extract was first fractionated on a phosphocellulose (P11) column. These three fractions used were then: 1) a 0.3M KCl step eluate from a DEAE-cellulose (DE52) column loaded with the flow through of the P11 column. This fraction contained TFIIA and was referred to as DE0.3. 2) A 0.5M KCl eluate of the P11 column was loaded on a double stranded DNA-sepharose column (31). The flow through (referred to as dsFT) contained RNA polymerase II, TFIIB, E, and F. 3) A 0.85M KCl eluate of the P11 column (referred to as P110.85) was used which contained TFIID.

In vitro transcription reactions

The three partially purified nuclear fractions were used in the transcription assay; 2 μl of DE0.3, 4 μl of dsFT, and 4 μl of

P110.85 except where indicated. The reaction mixture (25μl) contained 10 mM Tris-HCl (pH7.9), 40 mM HEPES-KOH (pH8.4), 60 mM KCl, 3 mM $MgCl_2$, 12% glycerol, 1 mM DTT, 0.5 mM ATP, GTP, CTP and UTP, 20 units placental RNase inhibitor (Promega), 25 ng pFC53X and 12.5 ng pML(s) plasmid DNA. Other templates were used as indicated and SRF was added in the amounts indicated. The standard incubation was at 30°C for 50 min. For preincubation experiments, the fractions were preincubated with all the components except nucleotides, with or without SRF at 30°C for 30 minutes. Nucleotides (and other fractions as indicated) were then added to start the transcription reaction and the incubation was continued for 20 minutes. After the reaction, 375 μl of 0.3 M sodium acetate (pH6.9) and 20 μg yeast RNA were added. After phenol and chloroform extraction and ethanol precipitation, the transcripts were analyzed by the S1 nuclease hybridization method as previously described (37). For S1 analysis of transcripts from pMC50, the plasmid was labelled at an EcoRI site in the CAT gene and digested with HindIII to generate a 330 nucleotide probe which was expected to give a 280 nucleotide protected fragment for correctly initiated transcripts. Where indicated, the levels of transcripts were quantitated using a PhosphorImager and ImageQuant software data analysis (Molecular Dynamics).

RESULTS

Inhibition of transcription by SRF

We used SRF synthesized in E. Coli (BSRF) to analyze transcriptional activation by SRF *in vitro*. The *in vitro* transcription reaction utilized three partially purified fractions from HeLa cell nuclear extracts containing RNA polymerase II and the general transcription factors TFIIA, -B, -D, -E and -F (38). The transcription reactions contained fosCAT reporter plasmids along with an adenovirus major late promoter plasmid (pML(s)) as a second template. The template plasmids used in this paper are diagrammed in figure 1. The reaction products were analyzed by S1 nuclease protection using two probes specific for either the fosCAT or adenovirus major late transcripts. While pML(s) was originally included as a TATA-only promoter control, it became apparent that it was activated by downstream promoter sequences (see below). As will be seen, it became useful to include a heterologous promoter that was inhibited but not activated by SRF.

Low amounts of BSRF activated transcription five fold from a fosCAT reporter gene (pFC53X) containing a high affinity binding site for SRF (Fig. 2A, lanes 5 and 6). No activation was observed using a template plasmid lacking the SRF binding site (pFC53; lanes 1 to 4). When higher amounts of BSRF were used, transcription from pFC53X was reduced (lanes 7 and 8) while there was no effect on transcription from pFC53 (lanes 3 and 4). As a control we also purified protein from bacteria containing the bacterial expression vector (without the SRF insert) in an identical manner to the purification of BSRF to determine if copurifying proteins or buffer contaminants might account for the effects observed on transcription. No effect was observed from this control preparation (data not shown). These results suggest that high amounts of SRF inhibit SRF-'activated' but not TATA-only 'basal' transcription.

We considered the possibility that SRF was only inhibiting by limiting high levels of transcription regardless of whether they were directed by 'basal' or 'activated' mechanisms. To rule this out we found conditions, varying the template concentration, where basal transcription levels (with pFC53) using high template concentrations (2 μg/ml) were similar to SRF-activated transcription levels (with pFC53X) using low template concentrations (0.25 μg/ml). Under these conditions, the 'basal' level was not affected by high SRF concentrations while the similar 'activated' level was still inhibited (data not shown).

It was also apparent that transcription from the adenovirus major late promoter plasmid was reduced by high amounts of

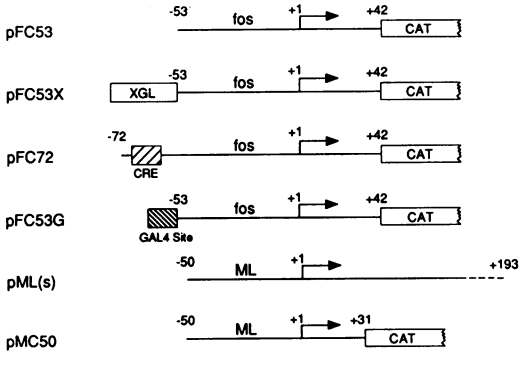

Figure 1. Template plasmids. The structures of the plasmids used as templates in the *in vitro* transcription reactions are shown. The regions derived from the c-fos and adenovirus major late (ML) promoters are indicated. In all cases there is a TATA element at −30. XGL is sequence from an oligonucleotide containing a high affinity SRF binding site. CRE is the cAMP response element at −60 in the c-fos promoter. The single GAL4 binding site in pFC53G is indicated. The constructs are described further in Methods. CAT: chloramphenicol acetyl transferase gene.

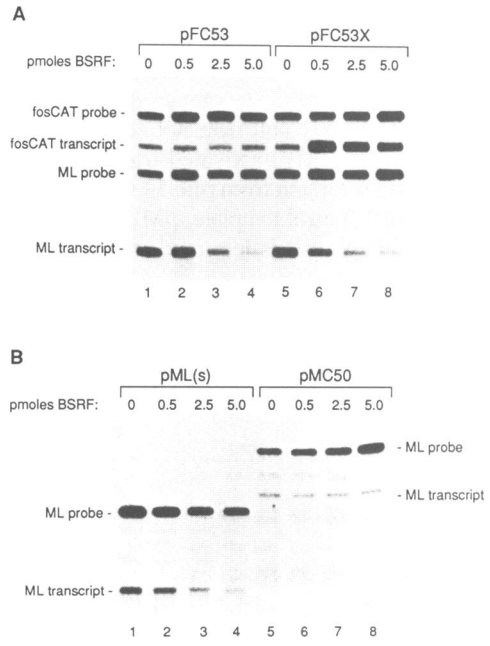

Figure 2. Inhibition of transcription by SRF. **A** and **B.** Increasing amounts of bacterially made SRF (BSRF) were added as indicated to *in vitro* transcription reactions containing 1 μg/ml of the plasmids indicated above the lanes. In **A)** 0.5 μg/ml pML(s) was also added to each lane. The transcripts were analyzed by S1 nuclease analysis using probes specific either for the fosCAT or major late (ML) transcripts. The positions of migration of the reannealed probes and specifically initiated transcripts on 4% polyacrylamide/7M urea gels are indicated.

SRF (Fig. 2). The adenovirus promoter plasmid, pML(s), contains −50 to +193 of the major late gene such that the binding site for an upstream activating protein (USF) is deleted (43). Nevertheless, there are reports of downstream binding proteins which regulate expression of the gene (44−47). To test whether pML(s) was, in fact, exhibiting downstream-activated rather than basal promoter activity, we made a construct, pMC50, which contained only −50 to +30 of the major late promoter fused to a CAT gene (Fig. 1). Transcription from this plasmid was reduced five fold compared to pML(s) and was not inhibited by high amounts of SRF (Fig. 2B). In contrast, pML(s) transcription was inhibited by SRF (Fig. 2A and B). Thus, in two cases SRF did not inhibit TATA-only transcription while it did inhibit transcription 'activated' by specific promoter binding factors. This experiment also demonstrates that inhibition by SRF does not require specific SRF binding sites on the plasmid DNA.

Inhibition of CREB- and GAL4-VP16-activated transcription

To further investigate whether SRF inhibits transcription activated by only certain gene-specific factors, or more generally by many specific factors, we tested whether SRF would inhibit transcription activated by CREB (also known as ATF) or a GAL4-VP16 fusion protein. CREB/ATF is a family of transcription factors which binds to cAMP response elements (CRE)(48,49). Some members of this family can mediate the response of genes to elevation in cAMP levels (50) while others can mediate activation of genes by the adenovirus E1A protein (51). CREB/ATF does not have a well classified transcriptional activation domain. There is a CRE in the fos promoter at −60 that raises the general level of transcription *in vivo* and can also mediate a further increase in transcription in response to cAMP (39,52). Since there is CREB present in our general transcription fractions, we have used reporter templates with (pFC72) and without (pFC53) CRE sites to indicate the activation of transcription by CREB (see Fig. 1). Transcription was elevated four to seven fold for pFC72 compared to pFC53 (Fig. 3A, compare lane 5 to lane 1 and data not shown). High amounts of SRF inhibited the CREB-activated transcription (lanes 5 to 8). Again, basal transcription from pFC53 was not inhibited (lanes 1 to 4). The internal control template, pML(s), was also inhibited in every case although this is less evident due to overexposure for this band.

The herpes viral VP16 protein is one of the strongest transcriptional activators known and contains an acidic activation domain (6). VP16 does not contain a DNA binding domain and activates transcription after association with a DNA binding protein such as Oct-1 (53−55). VP16 has also been fused to the GAL4 DNA binding domain, such that the chimeric protein is a potent activator of transcription from promoters containing GAL4 binding sites (6,42). We constructed such a promoter by cloning one GAL4 binding site at −53 in pFC53 to make pFC53G (Fig. 1). Transcription from this plasmid was activated by GAL4-VP16 (Fig. 3B, lanes 1 and 2). SRF neither activated nor inhibited basal transcription from pFC53G (data not shown). In the presence of GAL4-VP16, however, high amounts of SRF inhibited the GAL4-VP16 activated transcription (lane 10).

It should be noted that in the case of pML(s), pFC72, and pFC53G there are no specific SRF binding sites present on the plasmids. Thus, inhibition of transcription by SRF must be due either to excess SRF free in solution or SRF bound nonspecifically to DNA.

An SRF specific oligonucleotide blocks inhibition by SRF

In order to investigate the mechanism by which SRF inhibits transcription, we added a 52 bp double stranded oligonucleotide, XGL, which binds SRF with high affinity (37). This oligonucleotide will bind SRF such that it cannot bind to plasmid DNA and will therefore exist as a protein-DNA complex in solution. XGL was added to transcription reactions containing either low amounts of SRF (0.1 picomole) which activate transcription or high amounts of SRF (2.5 picomole) which inhibit transcription. Not surprisingly, XGL was able to inhibit SRF-activated transcription (Fig. 4, compare lanes 5 and 2). Surprisingly, however, XGL also blocked SRF's ability to inhibit transcription from pML(s) (compare lane 6 to lane 3). As a control, an oligonucleotide, XGLM, with two nucleotide mutations compared to the XGL sequence, was used. These mutations abolish binding of SRF to XGLM (40) and this control oligonucleotide had little effect on SRF-activated or -inhibited transcription (lanes 7 to 9). Thus, SRF bound to the XGL oligonucleotide was incapable of inhibiting activated transcription from pML(s). Similarly, we found that inhibition by SRF of GAL4-VP16-activated transcription was also specifically blocked by XGL (data not shown). We further found that longer restriction fragments (up to 320 bp) containing SRF binding sites were also able to block inhibition by SRF such that the effect of XGL was not dependent on its short length (data not shown). These results

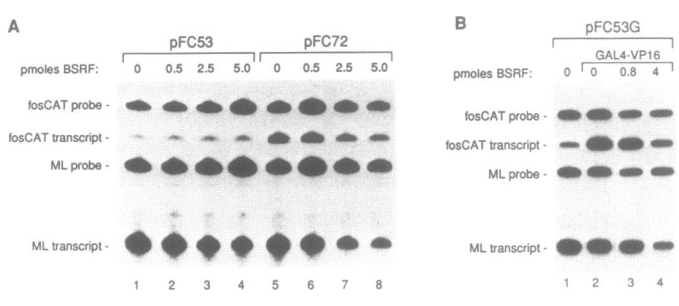

Figure 3. CREB- and GAL4-VP16-activated transcription are inhibited by high amounts of SRF. **A.** Increasing amounts of BSRF were added to transcription reactions containing the indicated plasmids (1 μg/ml) as well as pML(s)(0.5 μg/ml). **B.** As in **A** except that pFC53G was used and 0.8 pmole of GAL4-VP16 was added in lanes 2 to 4.

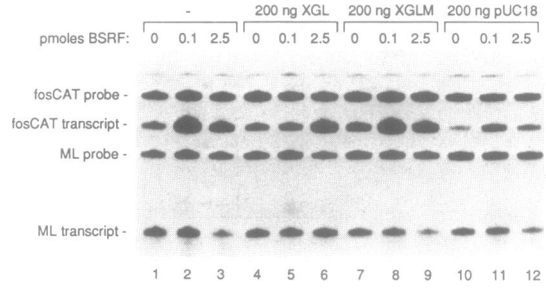

Figure 4. An oligonucleotide with an SRF binding site blocks inhibition of transcription by SRF. The transcription reactions contained pFC53X, pML(s) and the indicated amounts of BSRF. In addition, 200ng of XGL, XGLM, or pUC18 were added where indicated. XGL is an oligonucleotide which contains a high affinity SRF binding site. XGLM has two base pair changes relative to XGL and does not bind SRF.

suggest that to inhibit, SRF must either be free in solution or bound to plasmid DNA. The effect of XGL also further demonstrates that inhibition by SRF is not due to contaminants in the BSRF preparation.

Transcription from templates lacking SRF binding sites were still inhibited by SRF (Figs. 2B and 3). To test whether SRF must bind nonspecifically to the actual template plasmid, rather than to any plasmid DNA in the transcription reaction, we added excess pUC18 plasmid to the reaction. pUC18 (200 ng) was added in over a five fold excess compared to the template plasmids (25 ng for pFC53X and 12.5 ng for pML(s)). If SRF were inhibiting by binding nonspecifically to the template plasmid, it should not inhibit when excess pUC18 DNA is present since most of it should bind to pUC18. While pUC18 lowered the general level of transcription, it did not affect the inhibition of transcription from pFC53X or pML(s) by high amounts of SRF (Fig. 4, lanes 10 to 12). This suggests that SRF may inhibit transcription from one plasmid while binding to another plasmid in *trans*. This also leaves the possibility that SRF inhibits transcription when free in solution and not bound to DNA.

Effect of mutations in SRF on activation and inhibition

If SRF inhibits transcription while free in solution a mutant SRF which has lost the ability to bind DNA should still be able to inhibit transcription. For this purpose we tested a mutant which has a four amino acid insertion in the SRF DNA binding domain at amino acid 171 (Fig. 5D). This mutant, BSRF-In171, had no detectable binding to the XGL oligonucleotide in a gel mobility shift assay (data not shown). Its dimerization ability, however, was still intact. This was shown by denaturing and renaturing excess BSRF-In171 along with wild type (wt) BSRF. This resulted in a decrease of wt BSRF DNA binding activity suggesting that the mutant and wt proteins form an inactive heterodimer (data not shown). A mutant, BSRF-In206, with a four amino acid insertion in SRF's dimerization domain at amino acid 206 (Fig. 5D) neither bound to DNA nor dimerized by the above assays (data not shown).

These SRF mutants were tested for their ability to activate and inhibit transcription *in vitro*. As expected, due to its lack of DNA binding activity, BSRF-In171 was unable to activate transcription

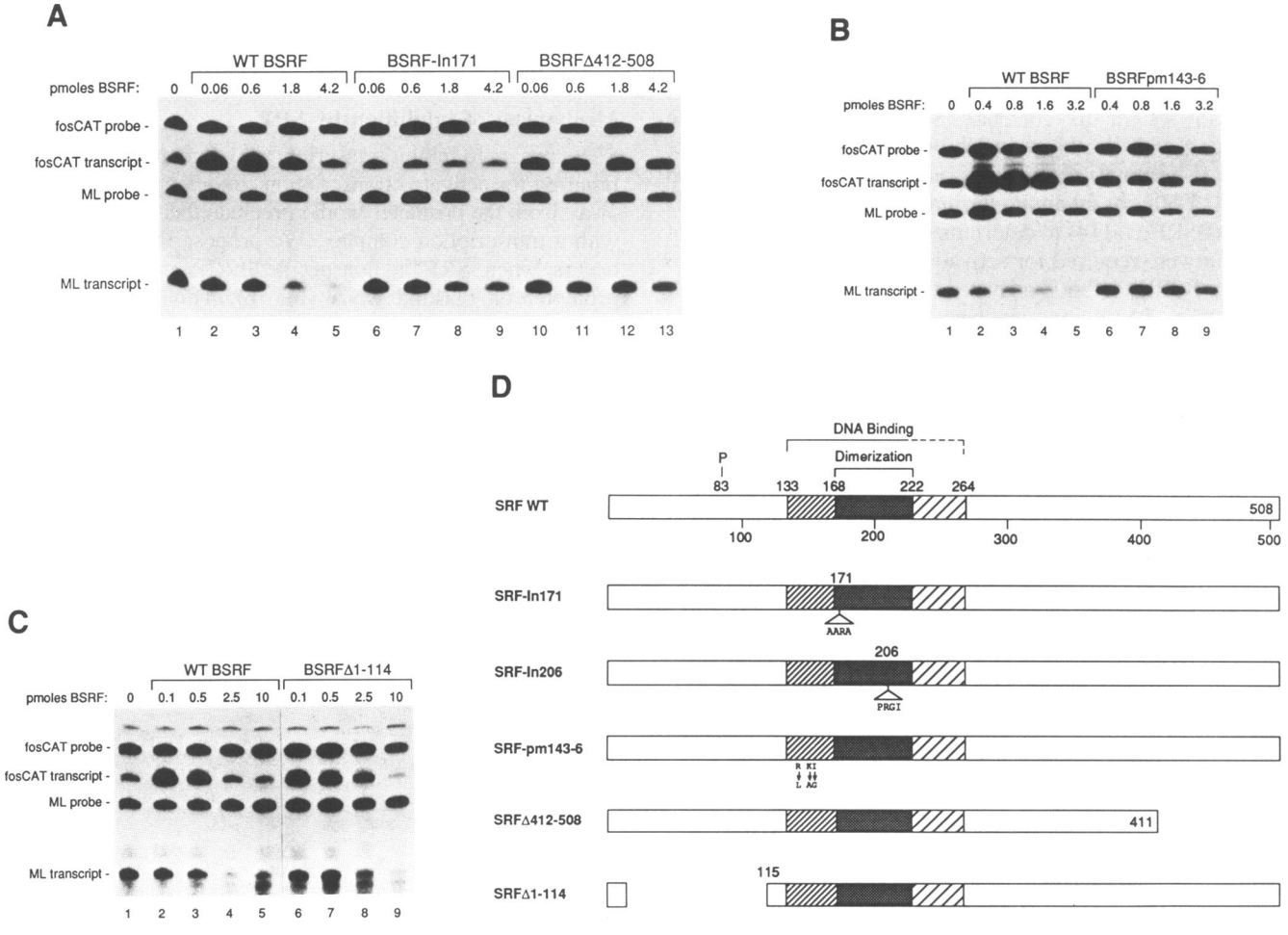

Figure 5. Effect of mutations in SRF on its ability to activate and inhibit transcription. **A–C.** Increasing amounts of wt or mutated SRF, as indicated above the lanes, were added to transcription reactions containing pFC53X and pML(s). The amounts added of the various SRF mutants were normalized to one another by immunoblot analysis. **D.** SRF mutations. The structure of wt and mutated SRFs are diagrammed. The regions required for DNA binding and dimerization as determined by Norman et al. (36) are indicated. Deletion of amino acids 222 to 264 diminished, but did not abolish, binding activity. In SRF-In171 and SRF-In206 the insertion of four amino acids *after* amino acid 171 and 206 are indicated. In SRF-pm143-6 the changes at positions 143, 145 and 146 are indicated. In SRFΔ1-114 the first 11 amino acids derived from the T7 phage gene 10 protein.

from pFC53X (Fig. 5A, lanes 6 to 9). High amounts of BSRF-In171 also did not inhibit transcription effectively from the pML(s) template. Similar results were observed with BSRF-In206 (data not shown). These results suggest that SRF free in solution is incapable of inhibiting transcription. Although there was some inhibition of major late transcription by BSRF-In171, this was small when compared to inhibition by the wild type protein (lanes 2 to 5). The small amount of inhibition observed with BSRF-In171 may be due to some residual ability to inhibit transcription or due to contaminants in the protein preparation. This latter possibility is supported by the observation that the basal fosCAT transcription was also slightly inhibited (lanes 8 and 9). As discussed above, preparations of wild type protein, in general, did not inhibit basal transcription (Fig. 2).

Since the linker insertions are relatively large mutations, it is possible that they affected the conformation of SRF rather than simply abolishing its DNA binding activity. For this reason we made a finer mutation which changed three amino acids in the amino terminal region of the DNA binding domain. We changed amino acids 143, 145 and 146 to create mutant SRF-pm143-6 (Fig. 5D). These amino acids were chosen because they are conserved among proteins distantly related to SRF in yeast (MCM1) and plants (deficiens) (51). SRF-pm143-6 had no detectable DNA binding activity in a gel mobility shift assay (data not shown). Similar to the linker insertion mutants it neither activated transcription nor inhibited transcription significantly compared to wt SRF (Fig. 5B).

We also sought to correlate SRF's ability to activate transcription with its ability to inhibit transcription. We made either a C-terminal deletion of amino acids 412 to 508 (BSRFΔ412-508) or an amino terminal deletion of amino acids 1 to 114 (BSRFΔ1-114) to determine whether these domains of the protein were required for activation of transcription *in vitro* (Fig. 5D). BSRFΔ412-508 activated transcription from pFC53X 2.8 fold (Fig. 5A, comparing lane 10 to lane 1) which was reproducibly lower than the 5.1 fold activation observed with wild type SRF (comparing lane 2 to lane 1). BSRFΔ412-508 was also defective in its ability to inhibit major late transcription. The highest amount tested (4.2 pmole) inhibited major late transcription by only 40% (lane 13 compared to lane 1) while wild type SRF inhibited major late transcription by 84% (lane 5 compared to lane 1). Thus, the abilities to activate and inhibit transcription appear to correlate, suggesting a common mechanism. It will be useful to have other mutants in the transcriptional activation domain to further correlate the activation and inhibition functions. Unfortunately, larger C-terminal deletion mutants were not stably produced in E. coli such that we were not able to test their abilities to affect transcription.

The amino terminal deletion mutant, BSRFΔ1-114, was able to activate and inhibit transcription just as well as wild type SRF (Fig. 5C). This suggests that the amino terminal domain of SRF is not involved in activation of transcription. The activity of BSRFΔ1-114 was surprising because the deletion removed a phosphorylation site for casein kinase II which increases SRF's DNA binding activity over 15 fold *in vitro* (40). We have found that this mutant has low DNA binding activity that cannot be increased by incubation with casein kinase II and ATP (41). Therefore, we expected that this mutant would activate transcription less efficiently. Further titration of SRF showed little difference in wild type SRF compared to BSRFΔ1-114 in the amount required for maximal transcriptional activation (data not shown). It is possible that the conditions of the *in vitro* transcription reaction do not distinguish between the low and high affinity forms of SRF. This could be because the SRF binding site concentration on the template plasmids is higher than the equilibrium association constant for even the low affinity (unphosphorylated) form of SRF.

DISCUSSION

We have used an *in vitro* transcription system derived from human HeLa cell nuclear extracts to demonstrate that SRF interacts with a factor(s), termed a coactivator, which is required for 'activated' but not 'basal' transcription. The lack of effect on basal, TATA-only, transcription suggests that a general transcription factor is not being affected by high levels of SRF. Rather, transcription activated by a diverse group of transcription factors was inhibited. High amounts of SRF inhibited transcription activated by itself, VP16, CREB/ATF, and downstream binding factors in the adenovirus major late gene. VP16 has an acidic activation domain (7) while the activation domains of the others have yet to be defined. We interpret the observation that SRF can inhibit transcription activated by all of these transcriptional activators as a reflection that they all interact with a common coactivator and thus use a common mechanism to increase transcription. Such a coactivator could be a novel factor or a specially modified form of one of the general transcription factors that is required for activated but not basal transcription.

Mechanism of inhibition by SRF

SRF appears to inhibit transcription by interacting with a factor required for activated transcription. Interaction of these factors away from the promoter would preclude them from associating with a transcription complex. We propose that this interaction occurs when SRF is nonspecifically bound away from the promoter on plasmid DNA (Fig. 6). This may be due to a requirement for the coactivator to also bind DNA when it interacts with a transcriptional activator. In support of this model, we found that SRF containing mutations that abolish its DNA binding

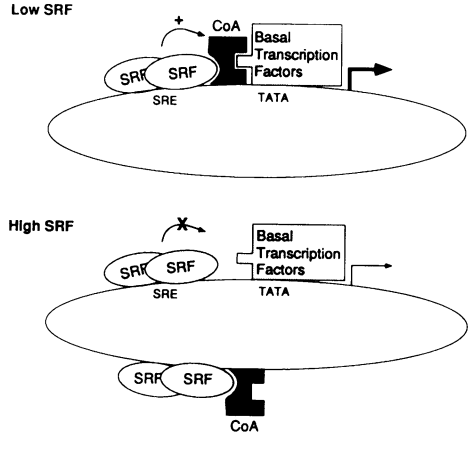

Figure 6. Model for inhibition of activated transcription by SRF. Low amounts of SRF bind specifically to the SRE at the promoter and interact with the coactivator (CoA) which activates transcription by the basal transcription factors (RNA polymerase II and TFIIA, -B, -D, -E and -F). High amounts of SRF also bind nonspecifically to plasmid DNA and complex with the coactivator such that not enough coactivator is available to mediate activation of transcription due to SRF bound at the promoter. See text for details.

activity would not inhibit. Moreover, SRF would also not inhibit when bound to an oligonucleotide. Thus SRF in solution (i.e. not bound to DNA) or bound to oligonucleotide DNA was not able to inhibit transcription from plasmid templates. The most plausible explanation for these results is that SRF inhibits transcription when bound to plasmid DNA.

The inhibition of activated transcription by SRF was not due to its specific DNA binding activity since templates without SRF binding sites were inhibited. Cryptic SRF binding sites also do not seem to be important since, of two closely related plasmids, one directing activated transcription (pFC72) was inhibited while a second, directing basal transcription (pFC53), was not. It also does not appear as though SRF must be bound to the actual template DNA. This is supported by the finding that activated transcription from pML(s) was still inhibited when excess pUC18 plasmid DNA was included in the reaction. Since neither of these contain SRF binding sites, there should be no preference for SRF to bind to either of these plasmids except for their abundance. The lack of inhibition of basal transcription also suggests that SRF is not inhibiting by simply binding to the promoter and preventing preinitiation complex formation. It is unlikely that such nonspecific binding would be enhanced by the multiple gene-specific activators we have tested.

It is surprising that SRF appears to be able to inhibit transcription when bound nonspecifically to plasmid DNA but not when bound to an oligonucleotide (52 bp) or longer (320 bp) DNA fragments. It is possible that the longer DNA fragments we used were still not long enough to allow for association of both SRF and the coactivator. Alternatively there may be sequence requirements for the coactivator to bind DNA that were not present on the DNA fragments tested. Finally, there could be differences in the way the factors associate with linear versus supercoiled plasmid DNA.

While the model proposed in figure 6 shows activation by low levels of SRF, the model also applies to activation by CREB, GAL4-VP16 or the adenovirus major late downstream activating proteins. In all these cases high levels of SRF would inhibit transcription by precluding the coactivator from binding at the promoter.

Our results contrast to those of Berger et al. (9) who studied inhibition of transcription by GAL4-VP16 in a yeast *in vitro* transcription system. They found that GAL4-VP16 inhibited both basal and activated transcription and that in the presence of a GAL4 binding site oligonucleotide only activated transcription was inhibited. As discussed above, we never observed inhibition of basal transcription and an SRF binding site oligonucleotide blocked the inhibition of activated transcription. These differences may be due to differences between SRF and GAL4-VP16 or to differences in the *in vitro* transcription systems used (yeast vs. human). It is interesting to note that SRF inhibited transcription activated by GAL4-VP16, suggesting that both factors interact with a common coactivator.

Recruitment of the coactivator to the preinitiation complex may be the crucial step towards activation of transcription by the gene-specific factors. Therefore, its isolation and reconstitution in an *in vitro* transcription system are essential for an understanding of the mechanism of transcriptional regulation. A coactivator could provide a bridge between a gene-specific activator and a general factor. Alternatively, it may be required in a larger complex contacting both/or either of these factors; for instance it might be required to complex with a general factor such that the general factor can function in an activated mode. One possible test to identify the coactivator in a mammalian system will be to assay fractions for their ability to overcome the inhibition of transcription described here. Our preliminary attempts have been surprisingly difficult because of the large number of general transcription factors. Adding excess fractions to overcome SRF's inhibition of transcription invariably results in adding more of one of the general transcription factors and results in elevated basal transcription. This makes the effects on activated transcription more difficult to interpret. Further fractionation and titration of the general transcription factors will be required to determine there requirement in activated transcription and to determine how a coactivator purifies. Since multiple coactivators might be titrated out by SRF, it will be interesting to see how purified coactivators relieve squelching.

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